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# FUNCTIONAL CONSEQUENCES OF CHEMICAL MODIFICATION OF THE SAXITOXIN BINDING SITE ON NEURONAL SODIUM CHANNELS.

Annual Summary Report
Period 9/1/85 through 8/31/86

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Robert J. French, Ph.D.

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Voltage-dependent, saxitoxin-(STX-) sensitive sodium channels from rat brain are being studied at the single channel level in artificial planar phospholipid bilayer membranes. In the presence of the activating neurotoxin, batrachotoxin, the channels are selective for sodium over other cations, are blocked by nanomolar extracellular STX and are more likely to be open at depolarized membrane potentials. The carboxyl-specific reagent, trimethyloxonium, renders the channels insensitive to STX, reduces the rate of sodium ion permeation through the channel and greatly decreases sensitivity to block by extracellular calcium. These results have led to the development of a rate theory model of ion permeation through the sodium channels in which the STX binding site, which contains a negatively-charged carboxyl residue, is an essential step in the ion permeation process. Experimental results supporting this model place the STX binding site close enough to the mouth of the channel pore to serve as this initial binding site for permeant ions when not occupied by blockers such as calcium and STX.

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## SUMMARY

Sodium channels from rat brain have been studied at the single channel level in planar phospholipid bilayer membranes. In the presence of the activating neurotoxin, batrachotoxin (BTX), the channels were selective for sodium over other cations and the probability of being open ($P_o$) was increased by membrane depolarization. Saxitoxin (STX) and tetrodotoxin (TTX) blocked the open channels inducing long-lived blocked periods corresponding to the toxin-bound state. Hyperpolarizing membrane potentials favored STX and TTX block with the $K_i$ for block increasing about e-fold for 40 mV change in potential. Voltage dependence of block by TTX (monovalent cation) and STX (divalent) was the same, leading to the conclusion that voltage dependence of block is due to a voltage-driven conformational change in the binding site structure. External divalent cations (e.g., $Ca^{++}$ and $Mg^{++}$) also blocked the open channels in a voltage-dependent manner, presumably by competing with permeant cation (e.g., $Na^{+}$) for one or more common sites in the channel pore. The carboxyl-methylating reagent, trimethyloxonium (TMO), applied from the extracellular side, abolished sensitivity to toxins, reduced single channel conductance and virtually eliminated block by external $Ca^{++}$. We conclude that the TMO-sensitive, toxin binding site may be an essential step in the ion permeation process. A three-site, four-barrier rate theory model has been developed to account for ion movement through the channel under a variety of experimental conditions assuming that permeant and blocking cations interact with the toxin binding site as they enter the channel. An alternative possibility is that the toxins bind relatively far from the mouth of the pore and inhibit ion movement by an indirect, alloseteric mechanism. One experiment to estimate the spatial relationship between the toxin binding site and the channel pore suggests that toxins bind close to the channel mouth. In order to examine the generality of voltage-dependent toxin block and TMO modification, we have begun to study some of the properties of veratridine (VER) activated sodium channels. Voltage-dependent gating and voltage-dependent block by STX and TTX were similar in VER and BTX-activated channels. The single channel conductance of VER-activated channels was only about 10 pS as compared to 25 - 30 pS for BTX-activated channels. These experiments should provide new information about the molecular structure of the STX binding site and its role in the functioning of the channels in the absence of toxins.

## FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. NIH 85-23, Revised 1985).

Animals are maintained in the Central Animal Facility of the University of Maryland School of Medicine. Animals are housed, cared for, and used strictly in accordance with USDA regulations. The University of Maryland School of Medicine Central Animal Facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. The program of animal care is directed by a full-time, specialty trained, laboratory animal veterinarian. This institution has an Animal Welfare Assurance on file with the NIH Office for Protection from Research Risks (OPRR), Assurance Number A0472.

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TABLE OF CONTENTS

## EXPERIMENTAL RESULTS

### A. Specific Aims of Original Proposal.

The original specific aims (listed below) have not changed. During the first year, substantial progress has been made on aims 1, 2, and 4. As a consequence of recent results and developments in the field, new experimental approaches to address these aims have been introduced (see sections D and E).

1. to determine the molecular basis of the voltage dependence of saxitoxin (STX) and tetrodotoxin (TTX) block of neuronal sodium channels.

2. to examine the effects of trimethyloxonium (TMO; a modifier of the negatively-charged toxin binding site) on ion permeation through the channels and on channel block by calcium and strontium.

3. to examine the effect of other carboxyl modifying reagents on ion permeation and block. Special attention will be paid to carbodiimides which render sodium channels insensitive to TTX.

4. to utilize the information in 1 - 3 above to derive a rate theory model for ion permeation through the channel.

5. to determine the rates of opening and closing of single sodium channels at varying membrane potentials and the effects of TMO treatment on these processes.

B. Publications and Scientific Meetings.

Publications.

Worley, J. F., R. J. French, and B. K. Krueger. 1986. Trimethyloxonium modification of single batrachotoxin-activated sodium channels in planar bilayers. J. Gen. Physiol. 87: 327-349.

Krueger, B. K., J. F. Worley III, and R. J. French. 1986. Block of sodium channels in planar lipid bilayers by calcium and guanidinium toxins. Are the mechanisms of voltage dependence the same? Annals of the New York Academy of Sciences. 479: 257-268.

French, R. J., J. F. Worley, W. F. Wonderlin, and B. K. Krueger. 1986. Three sites of calcium block in single sodium channels? Proceedings. of the Eighth Annual Conference of the IEEE Engineering in Medicine and Biology Society. 962-965.

Worley, J. F., R. J. French, and B. K. Krueger. Effects of divalent cations and membrane surface charge on ion permeation through single sodium channels from rat brain incorporated into planar lipid bilayers. in preparation.

Worley, J. F., B. K. Krueger, and R. J. French. Ion permeation, divalent ion block and chemical modification of single sodium channels. Description by a 4-barrier, single occupancy, rate theory model. in preparation.

Abstracts:

Worley, J. F., B. K. Krueger, and R. J. French. 1986. Methylation of a superficial carboxyl group in sodium channels reduces block by calcium, and unit conductance as well as abolishing block by saxitoxin. Biophys. J. 49: 42a.

Corbett, A. M., W. C. Zinkand, and B. K. Krueger. 1986. Saxitoxin (STX) and calcium interactions with purified sodium channels reconstituted in planar lipid bilayers. Soc. Neuroscience Abst. 12: 45.

Corbett, A. C., W. C. Zinkand, and B. K. Krueger. 1987. Activation of single neuronal sodium channels by veratridine and polypeptide neurotoxin in planar lipid bilayers. Biophys. J. 51: 435a.

Scientific Meetings.

Dr. Krueger presented an invited paper at the New York Academy of Sciences conference on "Tetrodotoxin, Saxitoxin, and the Molecular Biology of the Sodium Channel", 12/85.

Dr. Krueger presented a paper at the Biophysical Society meeting, San Francisco. CA, 2/86.

Dr. Krueger attended the Gordon Research conference on "Ion Channels in Muscle and other Excitable Membranes", 8/86.

C. Rate Theory Modelling.

Methods. All experiments described in this report have been carried out using membrane vesicles prepared from homogenates of rat brain that are enriched in $^{3}$H-STX binding sites (1). Sodium channels were incorporated into phosphatidylethanolamine-phosphatidylserine planar bilayers and studied in the presence of the activating toxin, batrachotoxin (BTX; 2,3).

Ion movement through selective channels can be described by assuming that each permeant ion must traverse a series of energy barriers as it moves through the channel pore. Between each pair of adjacent barriers is an energy minimum or well, occupancy of which is energetically relatively favorable. In general, according to such a so-called "rate theory" model, highly permeant ions encounter low energy barriers and shallow wells, whereas ions that encounter one or more very high barriers are impermeant and those that enter very deep wells are minimally permeant and actually block the flow of permeant ions. Voltage-dependent sodium channels have been modeled in this way (4) using a three-well (site), four-barrier rate theory model, however, the number of barriers and sites and their positions were chosen arbitrarily to allow for good fits to macroscopic current-voltage data. We have also used a four-barrier, three-site model to explain sodium and potassium movement through single BTX-activated sodium channels in planar bilayers (Worley, Wonderlin, Krueger and French, in preparation). Our model (Figure 1) was chosen to account for the following

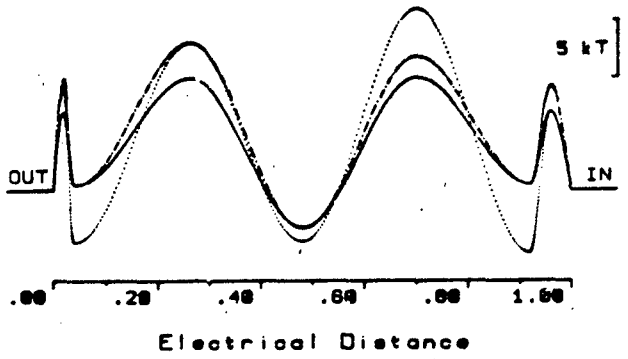


Figure 1. Energy profile for sodium (solid line), calcium (dotted line) and potassium (dashed line). In the rate theory model used in these studies, ions must traverse all four barriers to cross (permeate) the membrane through the channel.

experimental observations:

1. Trimethyloxonium (TMO) abolishes STX block and binding and profoundly affects sodium permeation and block by external calcium (5). Moreover, the STX biding site is probably located near the external surface of the channel (6). Thus, the saxitoxin binding site is assumed to be an initial binding site for entering cations and is represented by the external energy well.

2. External calcium blocks in a voltage-dependent manner with hyperpolarizing potentials favoring block (7). This is built into the model by placing a central site for either sodium or calcium reasonably deep within the channel so that entering cations experience a significant portion of the transmembrane electric field as they approach the site. Calcium blocks because it cannot

readily traverse the high barrier just to the inside of this site.

3. Internal calcium blocks sodium ion movement through the channels in a slightly voltage-dependent manner suggesting a third site, accessible from and quite close to the intracellular side of the channel (Worley, French and Krueger, in preparation).

The flux equations were solved (by computer) for each ion, to generate predicted single channel current-voltage relations in the absence and presence of calcium (Figure 2) and single channel conductance-sodium concentration

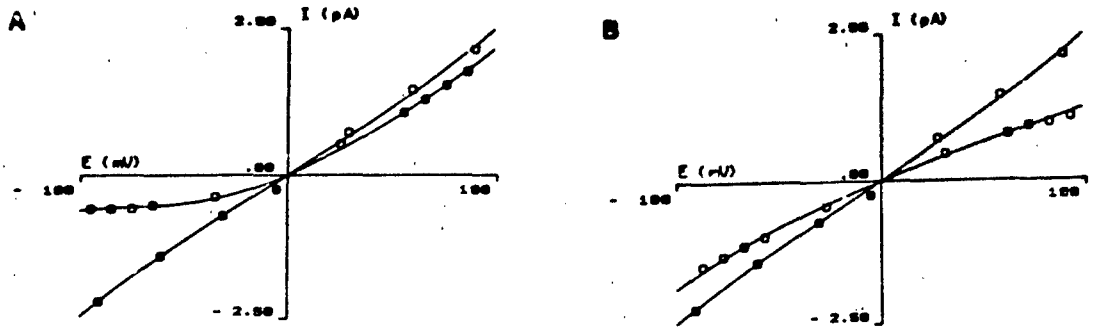


Figure 2. Single channel current vs. voltage relations for symmetrical 125 mM NaCl and 10 mM external (A) or internal (B) calcium. Lines are predictions from the rate theory model.

relations (Figure 3). Model parameters (energy levels) were varied to obtain a single set (Figure 1) that allowed satisfactory fits to all available data

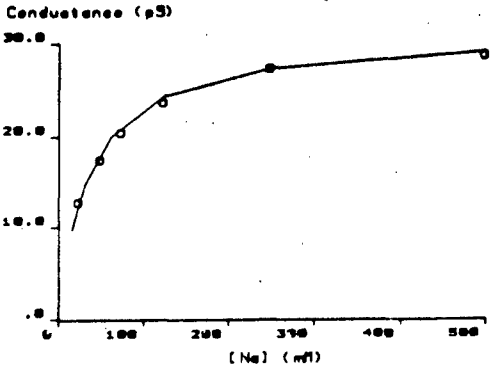


Figure 3. Single channel conductance vs. sodium concentration (symmetrical). The smooth line is the prediction from the rate theory model described above.

(Worley, French and Krueger, in preparation), including current-voltage relations and calcium block with "physiological" transmembrane ion concentration gradients (Figure 4). A critical test of this model was to mimic the effects of

TMC on both sodium permeation and calcium block by manipulating only the exter-

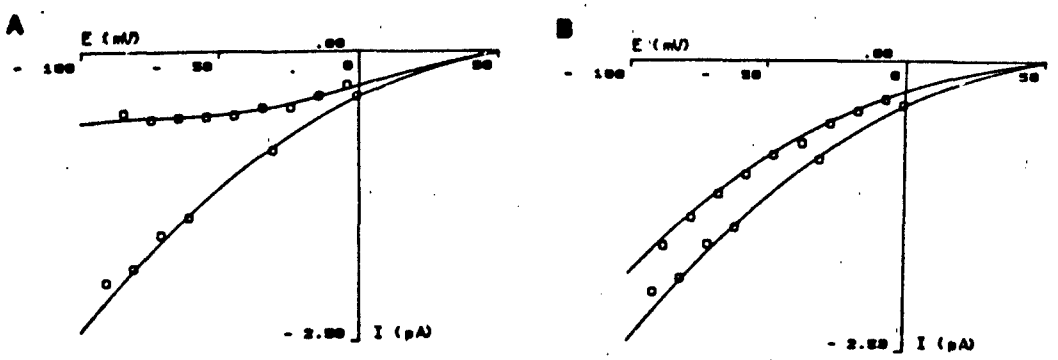


Figure 4. Single channel current vs. voltage relations for "physiological" ion gradients (125 mM Na/5 mM K outside; 125 mM K/5 mM Na inside) and 10 mM external (A) or internal (B) calcium. Lines are predictions from the rate theory model.

nal barrier and well. As shown in Figure 5, by making the outer barrier slightly higher and the outer well shallower for sodium and calcium, both the reduction in sodium conductance and the drastic decrease in potency of calcium block are

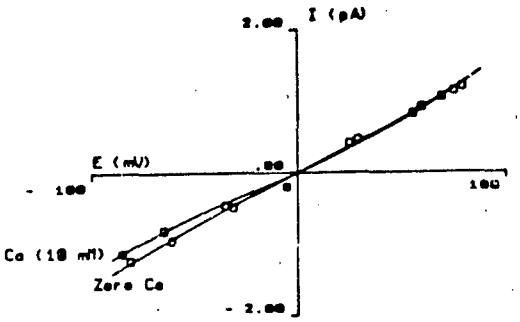


Figure 5. Single channel current vs. voltage relation for TMO-modified sodium channels in 125 mM symmetrical NaCl in the absence or presence of 10 mM external calcium. Compare with Figure 2A for unmodified channels.

accurately reproduced. It was concluded from these rate theory modelling studies that the experimental data are consistent with the saxitoxin binding site serving as a first and possibly essential step in the permeation of sodium through open sodium channels in the absence of blocking toxins.

D. The relationship between the saxitoxin binding site and the channel mouth. It is usually assumed that saxitoxin and tetrodotoxin block sodium channels by binding in or near the external mouth of the channel pore and sterically occluding entry of permeant ions (8). Indeed, it is implicit in the rate theory modelling described above that block by STX may be due to competition between permeant ions and toxin for an essential binding site in the permeation pathway (Worley, Wonderlin, Krueger and French, in preparation). With either of these mechanisms, toxin block may said to be by "occlusion" on the channel. Some experimental evidence leads to questioning this idea and the proposal that STX and TTX might bind at a site distant from the mouth of the channel pore and block by an "allosteric" mechanism involving a conformational change in the channel protein structure (9). The allosteric mechanism has been suggested because the presence of a negatively charged (TMO-modifiable) carboxyl group associated with the toxin binding site and located at the mouth of the channel pore would distort the shape of the conductance-sodium concentration relation (10). The conductance-sodium relation is well described by a simple, rectangular hyperbola (11 and Figure 3) suggesting that there is little negative charge at the channel entrance. An additional test of the binding site-channel mouth relationship is to examine the effects of external divalent cations (e.g., calcium) on the voltage dependence of STX block. The voltage-dependence of block by guanidinium toxins is thought to be due to a voltage-dependent conformational change in the structure of the toxin binding site rather that to the toxins entering the membrane electric field because both STX (a divalent cation) and TTX (monovalent) show the same voltage dependence (6,9,11,12). Rando and Strichartz (13) have suggested that this voltage-dependent block occurs only when the channels are open. In contrast to voltage-dependent guanidinium toxin block, calcium blocks open sodium channels with a similar voltage dependence because it binds to a site deep within the channel (see section C above). In light of this information, external calcium should reduce the voltage dependence of toxin block (via an electrostatic interaction between calcium and toxins, both cations, in their respective binding sites) if the toxin binding site is close to the calcium binding site inside the channel pore (e.g., at the entrance to the channel). Figure 6 shows that external calcium substantially reduces the voltage

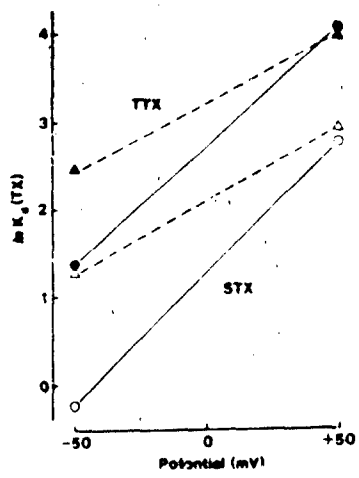


Figure 6. Semilog plot of $K_1$ for STX and TTX block vs. membrane potential in the absence (solid lines) and presence (dashed lines) of 10 mM extracellular calcium.

dependence of STX and TTX block, a result consistant with a toxin binding site at the extracellular mouth of the pore. Green et al. (9) have reported that external zinc, which exhibits similar voltage-dependent block of sodium channels, does not affect the voltage dependence of guanidinium toxin block. At the present time, the spatial relationship between the STX binding site and the channel entrance is unclear and there is not sufficient information to unequivocally distinguish between the "occlusion" and "allosteric" models for toxin block.

E. Activation of Na Channels by Veratridine.

Previously, all of the experiments on this project were carried out with BTX-activated sodium channels. By inhibiting sodium channel inactivation, BTX enables long, steady state recordings of single sodium channel currents at moderate bandwidths. In the absence of BTX, the channels would inactivate too fast to permit accurate resolution of unitary currents as is required for our work. It is possible that some of the properties we have observed are unique to BTX-activated channels and thus would not be representative of normal channels in vivo. We have therefore begun to study sodium channels in planar bilayers in the presence of veratridine (VER) an alkaloid toxin that also causes persistent activation of the channels. As shown in Figure 7, VER-activated channels are voltage-dependent (Fig. 7A) and are blocked by STX (7B).

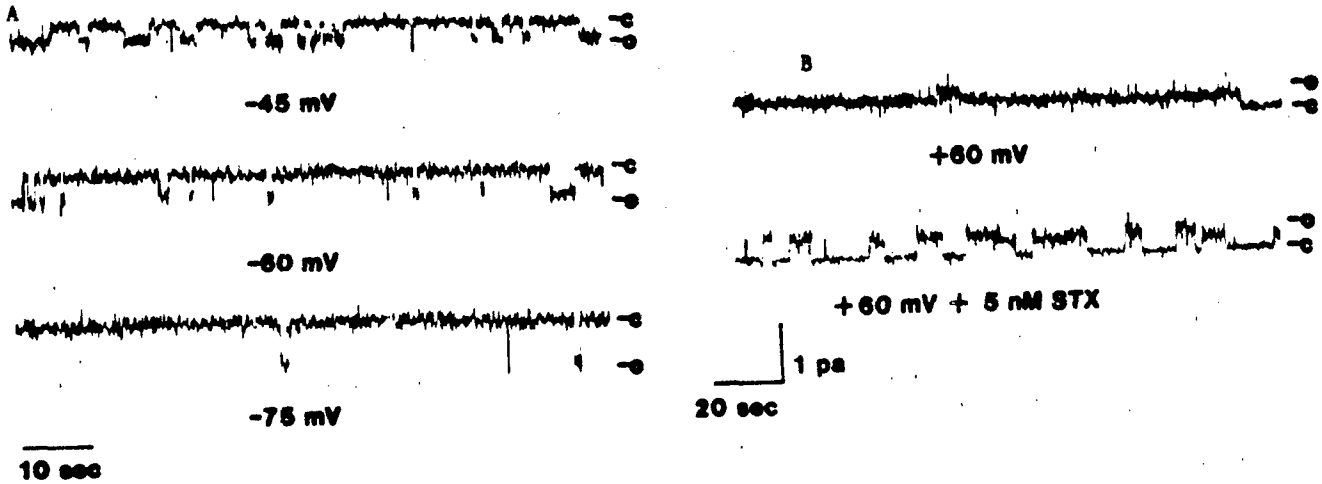


Figure 7. Veratridine-activated Sodium Channel. A. Single channel current fluctuations in the presence of 100 uM VER at various membrane potentials. B. Block of VER-activated channel by STX. Filtered at 40 Hz on playback.

Block of VER-activated channels by STX was voltage dependent (data not shown) as observed for BTX-activated channels (2,3,9,12). The most conspicuous difference between VER and BTX activated channels is that the single channel conductance of the VER-activated channels (10 pS, 250 mM NaCl) is only about 40% that of BTX-activated channels. The data in Figure 7 were obtained from partially purified, reconstituted sodium channels (14) that were about 30% pure; virtually identical channel properties, including VER activation, were observed with both native and purified channels (15). Garber and Miller (16) have observed similar properties of VER-activated sodium channels from skeletal muscle in planar bilayers.

Veratridine has been found to act on sodium channels synergistically with polypeptide toxins from scorpion and sea anemone venoms (17). As shown in Figure 8, we have found preliminary evidence for this synergism using a polypeptide

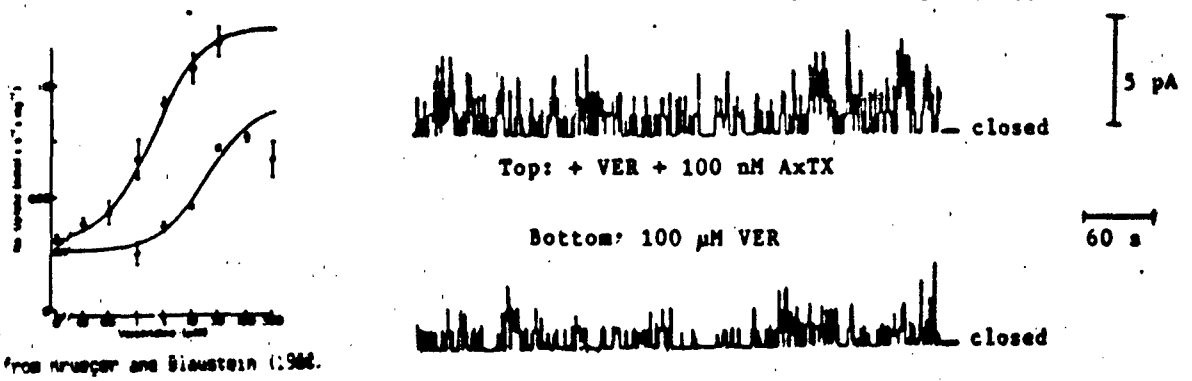


Figure 8. VER-AxTX Synergism. Left: Data from (17) showing the potentiation of VER-activated sodium influx into rat brain synaptosomes. Filled circles, VER alone; open circles, + 100 nM AxTX. All stimulated fluxes were STX sensitive. Right: single channel recording of VER-activated rat brain sodium channels in a planar bilayer. Addition of 100 nM AxTX greatly increases the probability that the channels will be open. Symmetrical 250 mM NaCl; filtered at 50 Hz on playback.

toxin from the sea anemone Anthropleura xanthogrammica (18, AxTX). In the absence of AxTX, there were several VER-activated channels with a low probability of being open. Addition of 100 nM AxTX greatly increased the open probability. It is interesting that the single channel conductance did not change upon AxTX activation. This potentiation may be a manifestation of the same VER-AxTX synergism seen in flux experiments (17).

Although the results are preliminary, it appears that, with the exception of the reduced single channel conductance, the properties of VER and VER+AxTX activated sodium channels, particularly those of the STX binding site, are similar to those of BTX activated channels.

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